University of Groningen

Inducible gene expression mediated by a repressor-operator system isolated from Lactococcus lactis bacteriophage r1t

Nauta, Arjen; Sinderen, Douwe van; Karsens, Harma; Smit, Egbert; Venema, Gerard; Kok, Jan

Published in:
Molecular Microbiology

DOI:
10.1111/j.1365-2958.1996.tb02477.x

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1996

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Nauta, A., Sinderen, D. V., Karsens, H., Smit, E., Venema, G., & Kok, J. (1996). Inducible gene expression mediated by a repressor-operator system isolated from Lactococcus lactis bacteriophage r1t. *Molecular Microbiology*, *19*(6), 1331-1341. https://doi.org/10.1111/j.1365-2958.1996.tb02477.x

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Molecular Microbiology (1996) 19(6), 1331–1341

Inducible gene expression mediated by a repressor–operator system isolated from *Lactococcus lactis* bacteriophage r1t

Arjen Nauta, Douwe van Sinderen,† Harma Karsens, Egbert Smit, Gerard Venema* and Jan Kok
Department of Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands.

Summary

A regulatory region of the temperate *Lactococcus lactis* bacteriophage r1t chromosome has been cloned and characterized. It encompasses the two divergently oriented genes *rro*, encoding the phage repressor, and *tec*. Both genes, of which the transcription start sites have been mapped, are preceded by consensus −35 and −10 promoter sequences. The region contains three 21 bp direct repeats with internal dyad symmetry which probably act as operators. Two of these repeats partially overlap the two promoter sequences. The distant third repeat is located within the *tec* coding sequence. Gel mobility shift assays demonstrated that Rro specifically binds to this sequence. To study possible transcriptional regulation of the region, a *lacZ* translational fusion with an open reading frame following *tec* was constructed. Under conditions that favour the lysogenic life cycle of r1t, β-galactosidase activity was very low. Expression of the *lacZ* fusion could be induced 70-fold by the addition of mitomycin C at a concentration which promotes the switch of r1t from the lysogenic to the lytic life cycle. In non-induced cells, promoter activity was repressed by Rro, as a frameshift mutation in *rro* resulted in constitutive expression of the *lacZ* gene fusion.

Introduction

Gene-expression systems in which the expression of a certain gene can be controlled can bypass the possible lethality of (heterologous) gene products. For the Gram-positive lactic acid bacterium *Lactococcus lactis* two inducible systems have been described so far. The *lac* promoter upstream of the lactococcal *lac* genes is regulated by the LacR repressor and can be used for lactose-inducible gene expression (van Rooijen *et al.*, 1992). Replacing glucose by lactose in the growth medium results in a sixfold induction of transcription of genes downstream of the *lac* promoter in cells containing an expression vector based on this system. In a further refinement of the system, it has recently been combined with a gene expression system based on the T7 RNA polymerase and its cognate promoter (Wells *et al.*, 1993). For that purpose, the gene encoding T7 RNA polymerase was placed under the control of the *lac* promoter. Upon growth on lactose of cells containing this system, the T7 RNA polymerase gene is expressed. As a result, expression of genes under the control of the T7 promoter is induced. A disadvantage of the T7/*lac* system is the fact that it is encoded by three different plasmids, which have to be introduced in *L. lactis*. In addition, the system relies upon heterologous DNA and is therefore not food grade, a prerequisite if it were to be applied in strains used in the food industry.

An easily manageable food-grade expression system allowing carefully controlled production of (heterologous) proteins (e.g. antimicrobials and enzymes involved in cheese-flavour development) would be of great interest. We set out to develop such a system on the basis of the regulatory region of a temperate *L. lactis* bacteriophage. Although many reports on the presence of temperate bacteriophages in lactococci have been published, little is known about the molecular basis for control and maintenance of the lysogenic state in these bacteria. A putative regulator gene, *bpi* (for BK5-T promoter inhibitor), of the temperate *L. lactis* subsp. *cremoris* phage BK5-T has been cloned and sequenced (Lakshmidevi *et al.*, 1990). The *bpi* gene product inhibited the activity of a number of BK5-T promoters. The mechanism by which the product of *bpi* operates is unknown. Recently, a putative repressor protein of the *L. lactis* bacteriophage Tuc2009 has been described (van de Guchte *et al.*, 1994). Although this protein shows significant homology with repressor proteins of other bacteriophages, its ability to control gene expression has not yet been demonstrated.

In this study we report the characterization of a regulatory region of the genome of the temperate small isometric-headed *L. lactis* subsp. *cremoris* bacteriophage r1t. The data presented show that a specific DNA fragment

Received 17 July, 1995; revised 9 November, 1995; accepted 20 November, 1995. †Present address: National Food Biotechnology Centre, University College Cork, Cork, Ireland. *For correspondence. Tel. (50) 3632093; Fax (50) 3632348.

© 1996 Blackwell Science Ltd

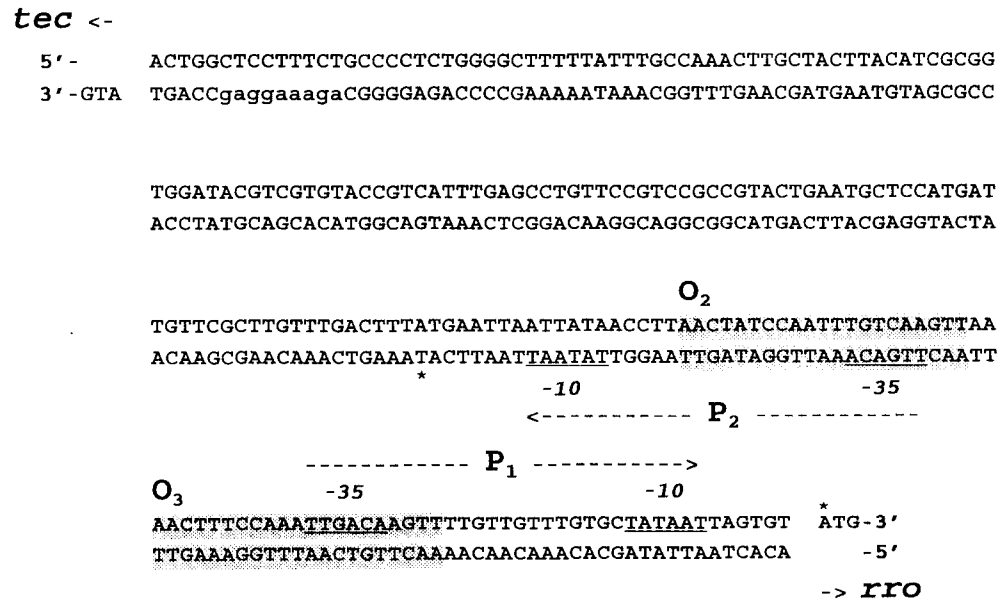

Fig. 1. Nucleotide sequence of the intergenic region between the oppositely directed genes *rro* and *tec*. The translational initiation codons are indicated. The −10 and −35 sequences of the two promoters P_1 and P_2 are underlined. The 21 bp direct repeats with dyad symmetry, O_2 and O_3, are shaded (for details see the text). The putative Shine–Delgarno (SD) sequence upstream of *tec* is indicated by lower-case letters. Asterisks indicate the 5′ ends of the transcripts produced from P_1 and P_2. This sequence appears in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Library under accession number U38906.

of bacteriophage r1t contains a gene (*rro*) that specifies a DNA-binding protein capable of repressing gene expression from a promoter on the same fragment. Using *lacZ* as a reporter gene, it is shown that this regulatory region of bacteriophage r1t can be exploited for inducible gene expression in *L. lactis*.

Results

The r1t regulatory region

We have recently determined the complete 33 350 bp nucleotide sequence of the temperate *L. lactis* subsp. *cremoris* bacteriophage r1t (see the accompanying paper by van Sinderen *et al.*, 1996). All of the identified open reading frames (ORFs) had the same orientation, except for a cluster of three consecutive ORFs (ORFs 1–3). ORF3 (designated *rro* hereafter) has an orientation opposite to that of ORF4 (*tec*) and ORF5. The non-coding region between the oppositely oriented *rro* and *tec* (Fig. 1) contained two divergently oriented sequences, P_1 and P_2, identical to the consensus promoter sequence used by the vegetative form of the *L. lactis* RNA polymerase (van de Guchte *et al.*, 1992).

The deduced amino acid sequence of *rro* shows significant similarity (78.8% identity) with the putative repressor CI of the *L. lactis* bacteriophage Tuc2009 (van de Guchte *et al.*, 1994b). Rro, which has a calculated molecular mass of 31.5 kDa, also shows similarity with the C-terminal parts of other phage-encoded repressor proteins such as the CI repressor of the *Escherichia coli* bacteriophage 434 (Nikolnikov *et al.*, 1984) and the C2 repressor of *E. coli* bacteriophage P22 (Sauer *et al.*, 1981). In addition, significant homology was found with various regulator proteins involved in SOS induction. In Fig. 2, the similarities of Rro with the phage repressors and the proteins LexA (Horii *et al.*, 1981) and DinR (Raymond-Denise and Guillen, 1991), which repress a set of damage-inducible genes in *E. coli* and *Bacillus subtilis*, respectively, are indicated. These similarities suggested that *rro* (for repressor of r-one) might specify the bacteriophage r1t repressor protein.

As most bacteriophage-specific repressor proteins contain a so-called helix-turn-helix motif involved in binding of the protein to its DNA target, we compared the deduced amino acid sequence of *rro* with a 'master set' of pre-aligned amino acid sequences known to adopt a helix-turn-helix conformation. By using the amino acid versus position score matrix (weight matrix) developed by Dodd and Egan (1990), which is based on the probability of a specific amino acid being present at a certain position within the motif, a score of 2157 was obtained for a stretch of 20 amino acids (Fig. 3). This score suggests that the sequence is likely to be involved in the binding of a specific DNA target.

The topological equivalent of the lambda *cro* gene, *tec*, could specify a protein of 80 amino acids with a calculated molecular mass of 9081 Da. Upstream of *tec*, a potential ribosome binding site is present that shows strong complementarity to the *L. lactis* 3′-16S rRNA sequence

Fig. 2. Alignment of Rro with the putative CI repressor of *L. lactis* bacteriophage Tuc2009, the C-terminal parts of the repressors CI and C2 of the *E. coli* bacteriophages 434 and P22, and the proteins LexA and DinR. Identical (*) and similar (#) amino acids between Rro and CI of Tuc2009 are indicated above the sequences, and amino acids that are identical (*) or similar (#) in all five sequences are indicated beneath the sequences. Conserved residues, between which autodigestion has been shown to take place in LexA (Little, 1993), are shaded. The putative helix-turn-helix of Rro is underlined.

($\Delta G = -19.4\,kcal\,mol^{-1}$) (Tinoco *at al.*, 1973; Chiaruttini and Milet, 1993). Although the method described above does not select a helix-turn-helix motif, the deduced amino acid sequence of *tec*, like that of *cro*, contains consensus amino acids at particular positions in helix-turn-helix motifs (Fig. 3).

P_1 and P_2 function as transcriptional start signals

The transcription initiation sites of *rro* and *tec* were determined by primer extension mapping. RNA was isolated from *L. lactis* cells harbouring either pIR12 or pIR13 (see below for a description of both plasmids).

protein	<			*helix*				>	<	*turn*	>	<					*helix*			>
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
r1t Rro	M	K	E	F	G	E	K	L	G	K	S	E	S	A	I	S	K	W	I	K
r1t Tec	A	A	N	I	A	E	V	I	G	V	D	T	L	D	V	I	F	V	L	K
Tuc2009 CI	Q	E	E	L	A	K	K	I	G	V	G	K	T	T	I	S	N	Y	E	V
λ CII	T	E	K	T	A	E	A	V	G	V	D	K	S	Q	I	S	R	W	K	R
ϕ80 gp30	H	K	V	L	A	E	K	V	G	V	T	P	Q	Q	A	I	N	M	L	K
P22 C2	Q	A	A	L	G	K	M	V	G	V	S	N	V	A	I	S	Q	W	E	R

Fig. 3. Alignment of the putative helix-turn-helix motifs in Rro and Tec with those in the putative transcriptional control protein CI of the *L. lactis* bacteriophage Tuc2009 and the repressors of the *E. coli* bacteriophages lambda (CII), ϕ80 (ϕ80 gp30), and P22 (C2). The latter three sequences were taken from Dodd and Egan (1990). Strongly conserved amino acids in the motif (Ala or Gly in position 5, Gly in position 9, and Ile or Val in position 15) are underlined.

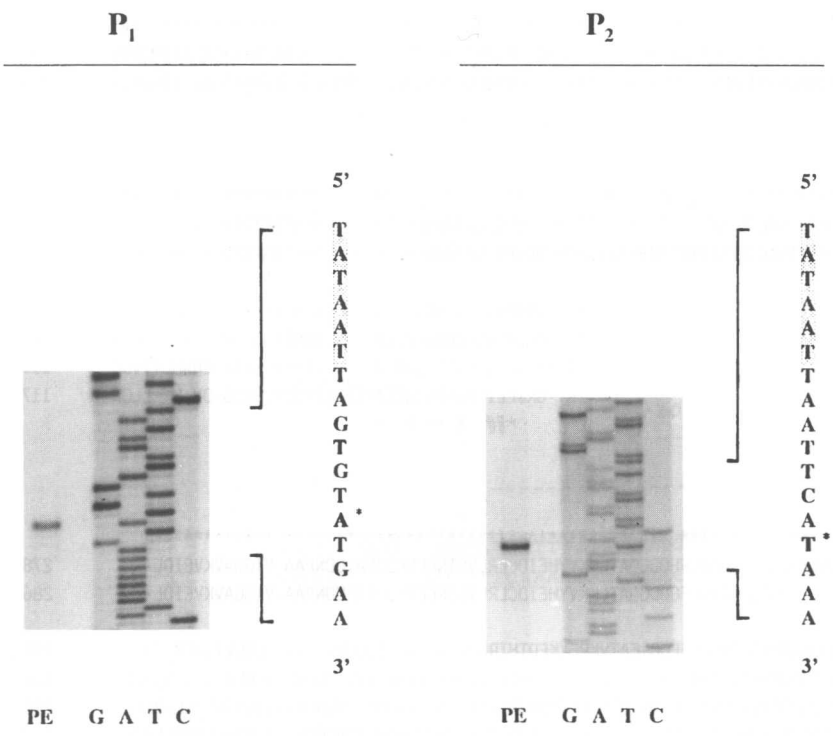

Fig. 4. Determination of the transcriptional start sites from P_1 and P_2 by primer extension mapping. Lanes PE show the extended products using primers RPE (P_1) and TPE (P_2). The nucleotide sequence ladders, obtained with the corresponding primers, were run in parallel. The relevant nucleotide sequences are indicated in the right-hand margins. The −10 sequences (shaded) and 5′ ends of the P_1 and P_2 transcripts (asterisks) are indicated.

The nucleotide sequences of the primers (RPE and TPE, respectively) that were used for the independent reverse transcriptions are depicted in the *Experimental procedures*. The studies confirmed the position of the two postulated promoters P_1 and P_2. The size of the extension products indicated that the transcription start site from P_1 is at the A at position 2757. The T at position 2846 specifies the 5′ end of the mRNA produced from P_2 (Fig. 4).

Rro binds to specific DNA sequences in the regulatory region

The intergenic region between *rro* and *tec* contains two almost perfectly matching 21 bp direct repeats with internal dyad symmetry, O_2 and O_3. The distance between both their centres constitutes 24 bp (Fig. 1). Because O_2 overlaps the −35 sequence of the promoter P_2, and O_3 overlaps that of P_1, these sequences may function as binding sites for the r1t repressor. A third putative operator site, O_1, is situated within the coding region of *tec* at a distance of 402 bp from O_2. Alignment of their six half-sites enabled the designation of an 11 bp-long consensus half-site (Fig. 5).

In order to determine whether Rro is able to bind DNA and specifically recognize one of the depicted 21 bp sequences, gel mobility shift assays were performed. The *rro* gene was amplified by the polymerase chain reaction (PCR) and placed under the control of the IPTG-inducible

operator	half-site sequence										
O_1	A	A	C	T	A	G	C	C	A	A	T
	A	A	C	T	T	G	A	C	A	A	A
O_2	A	A	C	T	A	T	C	C	A	A	T
	A	A	C	T	T	G	A	C	A	A	A
O_3	A	A	C	T	T	T	C	C	A	A	A
	A	A	C	T	T	G	T	C	A	A	T
consensus	A_6	A_6	C_6	T_6	T_4	G_4	C_3	C_6	A_6	A_6	T_3
					A_2	T_2	A_2				A_3
							T_1				

Fig. 5. Alignment of the six operator half-sites of O_1, O_2, and O_3. The 11 bp consensus half-site sequence, containing seven invariable nucleotides, is indicated.

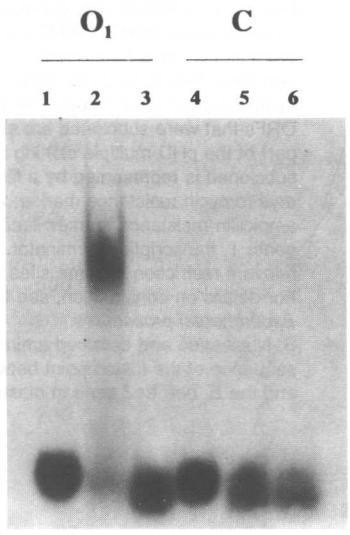

Fig. 6. Gel mobility-shift assays performed with the synthetic double stranded 21 bp DNA fragment O_1 (lanes 1–3), and a 21 bp negative-control fragment C (lanes 4–6). The [γ-^{32}P]-ATP-labelled DNA fragments were incubated for 15 min at room temperature with *E. coli* cell-free extracts either containing Rro (*E. coli* (pAG58SR)) or lacking Rro (*E. coli* (pAG58)). The samples were run through a non-denaturing 6% polyacrylamide gel. Lanes 1 and 4, no cell-free extract added; lanes 2 and 5, extract of *E. coli* (pAG58SR) added; lanes 3 and 6, *E. coli* (pAG58) extract added.

P_{spac} promoter in plasmid pAG58SR. Addition of cell-free extract of *E. coli* expressing *rro* to a 21 bp synthetic double-stranded O_1-site resulted in a lower mobility of this DNA fragment on a polyacrylamide gel as compared with the situation in which no cell-free extract or a cell-free extract not containing Rro (*E. coli* (pAG58)) was added (Fig. 6). Rro was unable to bind an arbitrary 21 bp fragment.

Expression of ORF5 is subject to repression by Rro

To study possible transcriptional regulation of the r1t region by Rro, a *lacZ* translational fusion was constructed with ORF5 in plasmid pIR12 (Fig. 7). In *L. lactis* LL302 carrying pIR12, very little β-galactosidase activity was observed (Fig. 8). To determine whether *lacZ* expression could be induced under conditions which normally induce the switch from the lysogenic to the lytic life cycle of r1t, the effect of mitomycin C on β-galactosidase activity was examined. After the addition of 1 μg ml^{-1} of mitomycin C to an *L. lactis* LL302 culture carrying pIR12, β-galactosidase activity increased considerably. Two and a half hours after the addition of mitomycin C, β-galactosidase activity had increased approximately 70-fold, indicating that ORF5 expression is transcriptionally regulated, presumably by promoter P_2. In order to examine whether the low *lacZ* expression in non-induced cells carrying plasmid pIR12 was established through repression of (P_2) promoter activity by Rro, pIR13 was constructed. Filling-in of the *Nco*I restriction-enzyme site of pIR12 resulted in a frameshift mutation and the introduction of two stop codons in *rro*. Therefore, cells containing pIR13 do not produce functional Rro. As can be seen in Fig. 8, such cells constitutively express *lacZ* at a high level. From these results we infer that the *rro* gene is required for repression of ORF5 transcription under conditions that favour the lysogenic state of the bacteriophage r1t.

Discussion

In this study we have identified a regulatory region of the temperate *L. lactis* bacteriophage r1t that is most probably involved in the control of the lysis–lysogeny 'decision'. On the basis of significant similarity of its deduced amino acid sequence with various repressor proteins, we assumed that *rro* specifies the bacteriophage r1t repressor protein. The *rro* gene is immediately preceded by promoter P_1. The identity of the −10 and −35 sequences of P_1 to the *L. lactis* vegetative promoter consensus is consistent with the fact that the lysogenic response to infection by a temperate phage requires the synthesis of a phage-encoded repressor (Ptashne, 1986). The repressor gene, therefore, is probably one of the first phage genes to be expressed after infection and, consequently, its expression should rely entirely upon phage-specific transcription initiation sequences recognized by the host RNA polymerase. The −35 and −10 sequences of P_2 are identical to those of P_1 but the presence in the latter of a −16 region in the spacer between both hexamers could account for possible bias in promoter utilization towards P_1. This 5′-RTRTG-3′ sequence, positioned one base upstream of the −10 hexamer of P_1, appears to be conserved in many promoters of Gram-positive bacteria (Moran *et al.*, 1982; van der Vossen *et al.*, 1987). For *B. subtilis*, it has been shown that this motif positively affects both promoter strength and utilization (Voskuil *et al.*, 1995).

On the basis of the N-terminal homology between Rro and the Tuc2009 CI repressor, we assume that the first possible initiation codon downstream of P_1 functions as the translation initiation signal of *rro*. As the adenine of this codon also functions as the transcription start site, this would be a situation analogous to that in the *E. coli* bacteriophages lambda and HK022. The P_{rm} transcripts of lambda and HK022 initiate at the A of the AUG start codons of the repressor genes and, therefore, lack 5′-leader and Shine–Delgarno (SD) sequences (Ptashne *et al.*, 1976; Cam *et al.*, 1991). For the lambda *c*I transcript it has been shown that translation depends on signals different from the conventional ribosome binding site (RBS) (Shean and Gottesman, 1992).

On the basis of the observed homology with specific portions of other phage-encoded repressor proteins, Rro seems to have a two-domain structure: an N-terminal portion

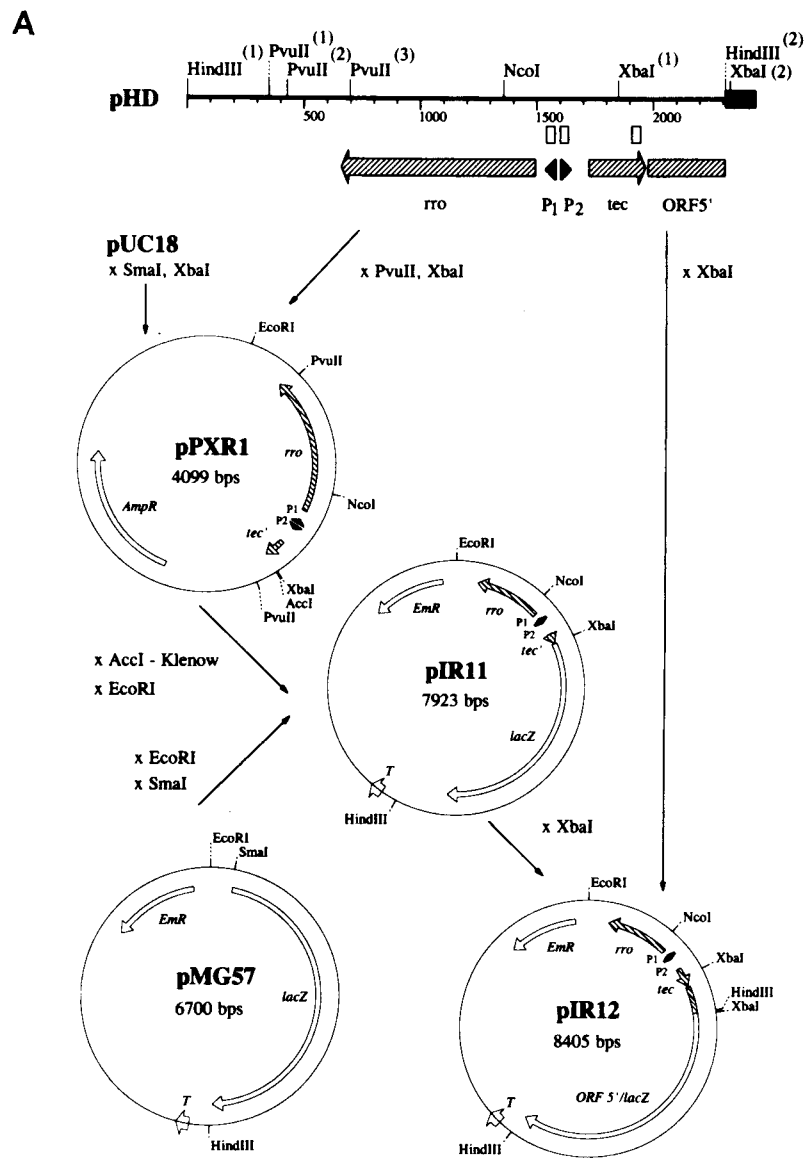

Fig. 7. A. Schematic representation of the construction of pIR12. ORF5, *rro*, *tec*, and the promotors P_1 and P_2 present on pHD are indicated. The three 21 bp direct repeats are symbolized by open boxes. The parts of the ORFs that were subcloned are shaded. The part of the pHD multiple cloning site that was subcloned is represented by a filled box. Em^R, erythromycin resistance marker; Amp^R, ampicillin resistance marker; *lacZ*, *E. coli lacZ* gene; T, transcription terminator. Only relevant restriction enzyme sites are indicated. For details on construction, see the *Experimental procedures*.
B. Nucleotide and deduced amino acid sequence of the fusion point between ORF5 and the *E. coli lacZ* gene in plasmid pIR12.

B

```
ACAATCCGAAGCACGGAGTACATGACGGATGCGAAGCTT  GCATGCCTGCAGGTCGACTCTAGAGTCGGG  GCCGTCGTTTTACAACGTCGTGAC
 T  I  R  S  T  E  Y  M  T  D  A  K  L     A  C  L  Q  V  D  S  R  V  G     A  V  V  L  Q  R  R  D

ORF 5' ->   -> pUC18 MCS                  -> codon 9 of E.coli lacZ
```

involved in binding to the operator sites, and a C-terminal domain which mediates dimer formation, dimer–dimer interaction, and self-cleavage. Upon DNA damage, self-cleavage of a phage repressor can occur via a RecA-mediated pathway which results in prophage induction. This autodigestion takes place in the so-called 'hinge region' connecting the N- and C-terminal domains at a specific site that is conserved in proteins that can undergo RecA-mediated cleavage (Little, 1993). These conserved amino acids are also present in the deduced amino acid sequence of Rro (Fig. 2). A putative DNA-binding motif is present in the N-terminal part of Rro. Like most of the bacteriophage-specified repressor proteins, Rro contains a so-called helix-turn-helix motif, suggesting that this stretch of amino acids is involved in binding to a specific DNA target, the operator (Pabo and Sauer, 1992). Despite

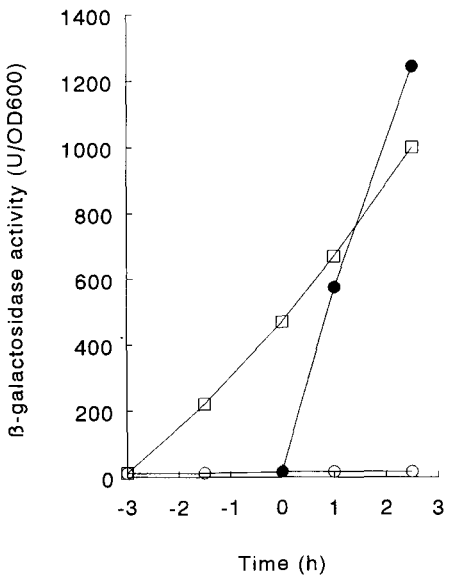

Fig. 8. Effect of mitomycin C on β-galactosidase activities measured in *L. lactis* LL302 carrying plasmid pIR12 (●) as a function of time. t_0 is the time point at which mitomycin C was added at a final concentration of 1 μg ml^{-1}. The β-galactosidase activities measured in cells carrying pIR12 and pIR13 without mitomycin C addition, are represented by (○) and (□), respectively. The time scale is in hours before and after t_0.

the high overall sequence similarity, the Rro and Tuc2009 CI repressor proteins differ in their N-terminal portions. It is therefore likely that both proteins recognize different DNA sequences.

Most of the bacteriophage operators described hitherto consist of imperfect symmetrical binding sites. In the case of phage lambda operators this twofold rotational symmetry reflects the two binding sites for the two monomers forming a repressor dimer (Pabo and Lewis, 1982). The subtle structural variation in the individual binding sites of the operators forms the basis for the differential relative affinities of CI and Cro for these sites (Ptashne, 1986). The three r1t operators differ slightly from each other and might function similarly. Indeed, Rro also binds O_2 and O_3, albeit with a lower affinity (results not shown). Of the 11 bp r1t consensus operator, seven nucleotides are invariable. Preliminary results indicated that Rro is unable to bind a 21 bp operator site of which one of these conserved nucleotides was substituted (results not shown).

The relative position of the putative operators of r1t clearly differs from that in bacteriophage lambda. Whereas in lambda all three operators are clustered in the non-coding area between *cI* and *cro*, enabling co-operative binding of repressor molecules, the centre of r1t O_1 is located 402 bp upstream from that of O_2. Such spacious arrangements of multiple operator sites have been demonstrated in the *E. coli* operons *gal* (Fritz *et al.*, 1983; Irani *et al.*, 1983), *araBAD* (Dunn *et al.*, 1984), *deo* (Vallentin-Hansen *et al.*, 1986) and *lac* (Eismann *et al.*, 1987). In all these cases, there is now accumulating evidence for a regulatory mechanism that involves co-operative binding of repressor to the distant sites through protein–protein contacts holding together a loop of intervening DNA (Schleif, 1992; Matthews, 1992). A similar situation has also been demonstrated for the *B. subtilis* phage φ105 (van Kaer *et al.*, 1987). Operator O_R3 of φ105 is located approximately 250 bp downstream of O_R2, within the proximal gene of the P_R transcription unit. Analogous to the regulatory regions of several other temperate bacteriophages, the region encompassing *rro* and *tec* is probably involved in the control of lysogeny of r1t. In this scheme, P_1 is apparently responsible for the establishment of the lysogenic state, whereas P_2 functions as the transcriptional start signal for the genes expressed during the lytic stage, being repressed during lysogenic growth. Indeed, expression of a *lacZ* fusion with ORF5, which is located downstream of P_2, was very low under conditions that favour the lysogenic life cycle of r1t. Expression of the *lacZ* fusion could be induced by the addition of 1 μg ml^{-1} mitomycin C, a concentration which in r1t promotes the switch to the lytic life cycle. Derepression of ORF5 transcription in non-induced cells in the absence of functional Rro (*L. lactis* (pIR13)) strongly suggests that Rro represses promoter P_2, because the region between *tec* and ORF5 does not provide space for a promoter, and because no promoter-like sequence could be discerned within *tec*.

On the basis of the results presented here, it would appear that the general strategy employed by r1t to control lysogeny is similar to that used by the lambdoid phages of *E. coli*. In bacteriophage lambda, *cro* is the first gene that is transcribed upon induction. Cro prevents transcription of the genes expressed during the lysogenic life cycle by binding to the same operator sites that CI recognizes. The order in which Cro binds to these sites, however, is opposite to that of CI. Although *tec* is the topological equivalent of lambda *cro*, it could specify a protein of approximately the same size as Cro, and the deduced amino acid sequence contains a putative DNA-binding α-helix–turn–α-helix motif, it still has to be clarified whether the *tec* gene product actually directs the bacteriophage into the lytic cycle and, if so, whether it is the functional equivalent of *cro*.

This study showed that a regulatory region of the lactococcal bacteriophage r1t can be exploited for inducible gene expression in *L. lactis*. Inducible gene expression in *E. coli* based on the temperature-sensitive CI repressor mutant CI857 has been extremely helpful as a simple means by which to overexpress (heterologous) genes in this organism. By analogy, such a system for lactococci would be valuable to modulate gene expression in this industrially important organism. Experiments are currently

Table 1. Bacterial strains, plasmids and bacteriophage used in this study.

Strain/Plasmid	Relevant features	Reference/ Source
Strain		
L. lactis subsp. *cremoris*		
LL302	MG1363 carrying the pWV01 *repA* gene on the chromosome	Leenhouts and Venema (1993)
E. coli		
MC1000	*araD139 Δlacx74 Δ(ara leu)7697 galU galK strA*	Casadaban and Cohen (1980)
Plasmid		
pUC18	Amp^R	Yanisch-Perron *et al.* (1985)
pHD	Amp^R; pUC18 derivative containing a 2.2 kb *Hin*dIII fragment of phage r1t	This work
pAG58	Amp^R Cm^R	Jaacks *et al.* (1989)
pAG58SR	Amp^R Cm^R; pAG58 derivative carrying *rro*	This work
pPXR1	Amp^R; pUC18 derivative carrying the 1428 bp *Pvu*II$^{(2)}$–*Xba*I$^{(1)a}$ fragment of pHD	This work
pMG57	Em^R; *lacZ* fusion vector, based on pWV01	van de Guchte *et al.* (1991)
pIR11	Em^R; pMG57 derivative carrying the 1450 bp *Eco*RI/*Acc*I fragment of pPXR1	This work
pIR12	Em^R; pIR11 derivative carrying the 482 bp *Xba*I fragment of pHD	This work
pIR13	Em^R; pIR12 derivative carrying a frameshift mutation in *rro*	This work
Bacteriophage		
r1t	Type P335, small isometric temperate lactococcal phage, isolated from *L. lactis* subsp. *cremoris* R1	Lowrie (1974)

a. For a key to the numbering of these restriction enzyme sites, see Fig. 7A.
Em^R, Amp^R, resistances to erythromycin and ampicillin, respectively.

under way to develop such a thermo-inducible gene-expression cassette from the regulatory region of r1t.

Experimental procedures

Bacterial strains, phage, plasmids, and media

The bacterial strains, phage and plasmids used in this study are listed in Table 1. *E. coli* was grown in TY broth (Rottlander and Trautner, 1970) or on TY broth solidified with 1.5% agar. *L. lactis* was grown in glucose M17 broth (Terzaghi and Sandine, 1975), or on glucose M17 agar. Erythromycin was used at 100 μg ml^{-1} and 5 μg ml^{-1} for *E. coli* and *L. lactis*, respectively. Ampicillin was added at a final concentration of 100 μg ml^{-1}. The chromogenic substrate Xgal (Sigma Chemical Co.) was added to plates at a final concentration of 40 μg ml^{-1}.

DNA techniques

Plasmid DNA was isolated essentially by the method of Birnboim and Doly (1979). Restriction enzymes, Klenow enzyme, and T4 DNA ligase were obtained from Boehringer Mannheim and used according to the instructions of the supplier. For nucleotide sequence analysis the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) was used with [α-^{35}S]-dATP (Amersham International) and the T7 sequencing kit (Pharmacia). Synthetic oligonucleotides were synthesized using an Applied Biosystems 381A DNA synthesizer. PCR was performed using Vent polymerase (New England Biolabs Inc.). Samples were heated to 94°C for 2 min, after which target DNA was amplified using a Thermocycler 60 PCR apparatus (BioMed) in 25 subsequent cycles under the following conditions: 94°C for 1 min; 50°C for 2 min; 73°C for 1 min. Helix-turn-helix motif predictions were performed according to Dodd and Egan (1990).

Plasmid constructions

A 2.2 kb *Hin*dIII fragment of bacteriophage r1t DNA, encompassing *rro*, *tec*, and parts of ORF2 and ORF5, was subcloned into the unique *Hin*dIII site of pUC18, resulting in the plasmid pHD. The 1428 bp *Pvu*II$^{(2)}$–*Xba*I$^{(1)}$ fragment of pHD was inserted into pUC18 restricted with *Sma*I and *Xba*I (Fig. 7A). The resulting plasmid, pPXR1, was restricted with *Acc*I and the 5′-protruding ends were blunted with Klenow enzyme. The linearized vector was subsequently digested with *Eco*RI and the fragment carrying *rro* and part of *tec* was ligated into *Eco*RI/*Sma*I-digested pMG57, resulting in plasmid pIR11. To restore *tec* and to fuse ORF5 in frame to the *lacZ* gene of *E. coli*, the 482 bp *Xba*I$^{(1)}$–*Xba*I$^{(2)}$ fragment of pHD was cloned into the unique *Xba*I site of pIR11, resulting in plasmid pIR12. The amino acid sequence at the fusion site between ORF5 and *lacZ* is shown in Fig. 7B. To introduce a frameshift mutation into *rro*, pIR12 was restricted with *Nco*I and the resulting 5′-sticky ends were filled in with Klenow enzyme. After self-ligation, an *Nsi*I restriction site was created in the resulting plasmid pIR13, as was confirmed by digestion with this enzyme. pIR12 and derivatives were propagated in *L. lactis* LL302 in order to increase the copy number of the plasmids by providing RepA *in trans*.

For the construction of pAG58SR, *rro* was amplified using PCR. An SD sequence was introduced upstream of *rro* in order to optimize expression of this gene in *E. coli*. The primers used for amplification had the following nucleotide sequences: 5′-ggggaagcttttggaggaattttgaaATGAAAAAAATACGACTACCTGAAATG-3′ (upper-case sequence corresponds to positions 2757 to 2731 of the r1t nucleotide sequence; EMBL/GenBank/DDBJ Nucleotide Sequence Data Library Accession Number U38906), and 5′-atagccggcatGCTTTTAACGAGAATCAAGACA-3′ (positions 1883 to 1904 of r1t). Following digestion with *Hin*dIII and *Sph*I (of which the sites are underlined in the PCR primers), the PCR fragment was subcloned in *Hin*dIII/*Sph*I restricted pAG58. In

Table 2. Oligonucleotides used for the construction of the 21 bp synthetic double-stranded DNA fragments.

Oligonucleotide	Nucleotide sequence	Position[a]
O_A	5′ AAC TAG CCA ATT TGT CAA GTT 3′	3227–3207
O_B	5′ AAC TTG ACA AAT TGG CTA GTT 3′	3207–3227
C_A	5′ GTC AAT CTA TTC AAT ACT GAT 3′	2598–2578
C_B	5′ ATC AGT ATT GAA TAG ATT GAC 3′	2578–2598

a. The positions correspond to the nucleotide sequences of bacteriophage r1t (EMBL/GenBank DDBJ Nucleotide Sequence Data Library Accession Number U38906).

pAG58SR *rro* is under the control of the IPTG-inducible P_{spac} promoter. The nucleotide sequence of *rro* was verified by nucleotide sequencing.

Transformation

E. coli was used as cloning host and transformed using the method of Mandel and Higa (1970). Plasmids were introduced in *L. lactis* LL302 by electrotransformation (Holo and Nes, 1989) with the modifications suggested by Leenhouts and Venema (1993).

IPTG- and mitomycin C induction

Overnight cultures were diluted 100-fold in fresh glucose M17 medium (*L. lactis*) or TY medium supplemented with 0.5% glucose (*E. coli*) and grown until the cultures had reached an OD_{600} of 0.3 (*L. lactis*) or 0.5 (*E. coli*). Mitomycin C (Sigma Chemical Co.) was then added to the *L. lactis* culture at a final concentration of 1 μg ml^{-1}. IPTG was added to *E. coli* at a final concentration of 5 mM. Before the addition of IPTG, *E. coli* cells were collected by centrifugation and resuspended in an equal volume of TY medium without additional glucose.

Assay of β-galactosidase activity

Cells from 5 ml of culture were collected by centrifugation and resuspended in 1 ml of cold Z-buffer (Miller, 1972). Glass beads (0.1 mm in diameter) were added and the cells were disrupted at 4°C for 15 min using a 'Shake it, Baby' cell disrupter (Biospec Products). Glass beads and cells debris were removed by centrifugation for 5 min in an Eppendorf centrifuge. Equal amounts of cell-free extract (0.5 ml) and cold Z-buffer were mixed and the β-galactosidase activity per OD_{600} was determined essentially as described by Miller (1972).

Gel retardation experiments

Gel retardation experiments were carried out essentially as described by Ebbole and Zalkin (1989). For the preparation of cell-free extracts of *E. coli*, cells were collected by centrifugation 3 h after the addition of IPTG, and resuspended in 1 ml of breaking buffer (20 mM Tris pH 8.0, 200 mM NaCl, and 1 mM EDTA). Glass beads (0.1 mm in diameter) were added and the cells were disrupted at 4°C for 10 min using a 'Shake it, Baby' cell disrupter (Biospec Products). Glass beads and cell debris were removed by centrifugation for 5 min at 20 000 × *g*. Lysates were stored at −80°C. Oligonucleotides that were used for the construction of the 21 bp synthetic double-stranded DNA fragments are indicated in Table 2. The single-stranded synthetic oligonucleotides O_A and C_A (30 ng) were end-labelled with polynucleotide kinase (Pharmacia) in the appropriate buffer for 1 h at 37°C using 30 μCi [γ-^{32}P]-ATP (Amersham). Labelled oligonucleotides were collected by Sephadex G-50 chromatography (Wall *et al.*, 1988) and purified by phenol/chloroform extraction. After ethanol precipitation in the presence of 1 μg poly(dI–dC) and 1 mM $MgCl_2$ the labelled oligonucleotides were annealed with their 'cold' synthetic complementary DNA fragments, O_B and C_B (120 ng), in 1× HIN (6 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 50 mM NaCl, and 1 mM dithiothreitol (DTT)) by heating for 2 min at 90°C, after which the samples were cooled to 4°C. Binding was carried out in 20 μl reaction volumes containing 20 mM Tris-HCl pH 8.0, 20% (v/v) glycerol, 1 mM EDTA pH 8.0, 200 mM KCl, 1 mM DTT, 100 μg ml^{-1} acetylated bovine serum albumin, 100 μg ml^{-1} poly(dI–dC), labelled DNA fragment (approximately 75 pg), and cell lysate (1–9 μg). After incubation for 15 min at room temperature, the samples were loaded onto a 6% polyacrylamide gel containing 3% glycerol. Gels were run in TAE buffer (0.04 M Tris-acetate pH 7.5, 2 mM EDTA) at 150 V for 1.5 h and dried. The gels were used for autoradiography at −70°C using Kodak XAR-5 film and intensifying screens.

Primer extension analysis

RNA was isolated from exponentially growing *L. lactis* cells carrying pIR12 or pIR13, as described by van Asseldonk *et al.* (1993). Synthesis of cDNA was performed using the Boehringer reverse transcription–PCR (RT–PCR) (AMV) Kit essentially as described by the supplier. mRNA (2 μg) of both strains was reverse transcribed using 50 ng of the synthetic oligonucleotides RPE (5′-CTTCAACCATGGGACTTC-TAACCCCTTTTATCCATTTCG-3′; positions 2613–2651 of r1t nucleotide sequence) or TPE (5′-CCTGAAGAGTCTAA-TAACTCATCTAGTGGCTC-3′; positions 3039–3008 of r1t), and 10 μCi [^{35}S]-dATP (Amersham). Reaction mixtures were incubated for 10 min at 48°C and 46°C, respectively, followed by another 10 min in the presence of 0.5 mM dATP, after which loading buffer was added. Internally labelled extended products were separated by electrophoresis on a 6% polyacrylamide urea gel and analysed by autoradiography.

Acknowledgements

We thank the Unilever Research Laboratory, Vlaardingen, for financial support; Dr A. M. Ledeboer, Dr W. Musters, L. Hamoen, and B. J. Haijema for helpful discussions; and H. Mulder for photography. J.K. is the recipient of a fellowship

of the Royal Netherlands Acadamy of Arts and Sciences (KNAW).

References

van Asseldonk, M., Simons, A., Visser, H., de Vos, W.M., and Simons, G. (1993) Cloning, nucleotide sequence and regulatory analysis of the *Lactococcus lactis dnaJ* gene. *J Bacteriol* **175:** 1637–1644.

Birnboim, H.C., and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucl Acids Res* **7:** 1513–1523.

Cam, K., Oberto, J., Weisberg, R.A. (1991) The early promoters of bacteriophage HK022: contrasts and similarities to other lambdoid phages. *J Bacteriol* **173:** 734–740.

Casadaban, M.J., and Cohen, S.N. (1980) Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. *J Mol Biol* **138:** 179–207.

Chiaruttini, C., and Milet, M. (1993) Gene organization, primary structure and RNA processing analysis of a ribosomal RNA operon in *Lactococcus lactis*. *J Mol Biol* **230:** 57–76.

Dodd, I.B., and Egan, J.B. (1990) Improved detection of helix-turn-helix DNA-binding motifs in protein sequences. *Nucl Acids Res* **18:** 5019–5026.

Dunn, T.M., Hahn, S., Ogden, S., and Schleif, R.F. (1984) An operator at −280 base pairs that is required for repression of *ara*BAD operon promoter: addition of DNA helical turns between the operator and promoter cyclically hinders expression. *Proc Natl Sci USA* **81:** 5017–5020.

Ebbole, D.W., and Zalkin, H. (1989) Interaction of a putative repressor protein with an extended control region of the *Bacillus subtilis pur* operon. *J Mol Biol* **264:** 3553–3561.

Eismann, E., von Wilcken-Bergmann, B., and Müller-Hill, B. (1987) Specific destruction of the second *lac* operator decreases repression of the *lac* operon in *Escherichia coli* fivefold. *J Mol Biol* **195:** 949–952.

Fritz, H.J., Bicknäse, H., Gleumes, B., Heibach, C., Rosahl, S., and Ehring, R. (1983) Characterization of two mutations in the *Escherichia coli galE* gene inactivating the second galactosidase operator and comparative studies on repressor binding. *EMBO J* **2:** 2129–2135.

van de Guchte, M., Kok, J., and Venema, G. (1991) Distance-dependent translational coupling and interference in *Lactococcus lactis*. *Mol Gen Genet* **227:** 65–71.

van de Guchte, M., Kok, J., and Venema, G. (1992) Gene expression in *Lactococcus lactis*. *FEMS Microbiol Rev* **88:** 73–92.

van de Guchte, M., Daly, C., Fitzgerald, G.F., and Arendt, E.K. (1994) Identification of the putative repressor-encoding gene *c*I of the temperate lactococcal bacteriophage Tuc2009. *Gene* **44:** 93–95.

Holo, H., and Nes, I.F. (1989) High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. *Appl Environ Microbiol* **55:** 3119–3123.

Horii, T., Ogawa, T., Nakatani, T., Hase, T., Matsubara, H., and Ogawa, H. (1981) Regulation of SOS functions: purification of *E. coli* LexA protein and determination of its specific site cleaved by the RecA protein. *Cell* **27:** 515–522.

Irani, M.H., Orosz, L., and Adhya, S. (1983) A control element within a structural gene: the *gal* operon of *Escherichia coli*. *Cell* **32:** 783–788.

Jaacks K.J., Healy, J., Losick, R., and Grossman, A.D. (1989) Identification and characterization of genes controlled by the sporulation-regulatory gene *spo0H* in *Bacillus subtilis*. *J Bacteriol* **171:** 4121–4129.

van Kaer, L., Van Montagu, M., Dhease, P. (1987) Transcriptional control in the *Eco*R1-F immunity region of *Bacillus subtilis* phage φ105. Identification and unusual structure of the operator. *J Mol Biol* **197:** 55–67.

Lakshmidevi, G., Davidson, B.E., and Hillier, A.J. (1990) Molecular characterization of promoters of the *Lactococcus lactis* subsp. *cremoris* temperate bacteriophage BK5-T and identification of a phage gene implicated in the regulation of promoter activity. *Appl Environ Microbiol* **56:** 934–942.

Leenhouts, K.J., and Venema, G. (1993) Lactococcal plasmid vectors. In *Plamids, A Practical Approach*. Hardy, K.G. (ed.). Oxford: Oxford University Press, pp. 65–94.

Little, J.W. (1993) LexA cleavage and other self-processing reactions. *J Bacteriol* **175:** 4943–4950.

Lowrie, R.J. (1974) Lysogenic strains of group N lactic Streptococci. *Appl Microbiol* **27:** 210–217.

Mandel, M., and Higa, A. (1970) Calcium-dependent bacteriophage DNA infection. *J Mol Biol* **53:** 159–162.

Matthews, K. (1992) DNA looping. *Microbiol Rev* **56:** 123–136.

Miller, J. (1972) In *Experiments in Molecular Genetics*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Moran, Jr, C.P., Lang, N., LeGrice, S.F.J., and Losick, R. (1982) Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. *Mol Gen Genet* **186:** 339–346.

Nikolnikov, S., Posfai, G., and Sain, B. (1984) The construction of a versatile plasmid vector that allows direct selection of fragments cloned into six unique sites of the *c*I gene of coliphage 434. *Gene* **30:** 261–265.

Pabo, C.O., and Lewis, M. (1982) The operator-binding domain of lambda repressor: structure and DNA recognition. *Nature* **298:** 443–447.

Pabo, C.O., and Sauer, R.T. (1992). Transcription factors: structural families and principles of DNA recognition. *Annu Rev Biochem* **61:** 1053–1095.

Ptashne, M. (1986) *A Genetic Switch*. Cambridge, Massachusetts: Cell Press and Blackwell Scientific Publications.

Ptashne, M., Backman, K., Humayun, M.Z., Jeffrey, A., Maurer, R., Meyer, B., and Sauer, R.T. (1976) Autoregulation and function of a repressor in bacteriophage lambda. *Science* **194:** 156–161.

Raymond-Denise, A., and Guillen, N. (1991) Identification of *dinR*, a DNA damage-inducible regulator gene of *Bacillus subtilis*. *J Bacteriol* **173:** 7084–7091.

van Rooijen, R.J., Gasson, M.J., and de Vos, W.M. (1992) Characterization of the promoter of the *Lactococcus lactis* lactose operon: contribution of flanking sequences and LacR repressor to its activity. *J Bacteriol* **174:** 2273–2280.

Rottlander, E., and Trautner, T.A. (1970) Genetic and transfection studies with *Bacillus subtilis* phage SP50. *J Mol Biol* **108:** 47–60.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA

sequencing with chain termination inhibitors. *Proc Natl Acad Sci USA* **74:** 5463–5467.

Sauer, R.T., Pan, J., Hopper, P., Hehir, K., Brown, J., and Poteete, A.R. (1981) Primary structure of the phage P22 repressor and the gene *c2*. *Biochemistry* **20:** 3591–3598.

Schleif, R.F. (1992) DNA looping. *Annu Rev Biochem* **61:** 199–223.

Shean, C.S., and Gottesman, M.E. (1992) Translation of the prophage λ *cI* transcript. *Cell* **70:** 513–522.

van Sinderen, D., Karsens, H., Kok, J., Terpstra, P., Ruiters, M.H.J., Venema, G., and Nauta, A. (1996) Sequence analysis and molecular characterization of the temperate lactococcal bacteriophage r1t. *Mol Microbiol* **19:** 1343–1355.

Terzaghi, B.E., and Sandine, W.E. (1975) Improved medium for lactic streptococci and their bacteriophages. *Appl Microbiol* **29:** 807–813.

Tinoco, Jr, I., Bore, P.N., Dengler, B., Levine, M.D., Uhlenbeck, O.C., Crothers, D.M., and Gralla, J. (1973) Improved estimation of secondary structure in ribonucleic acids. *Nature* **246:** 40–41.

Vallentin-Hansen, P., Albrechtsen, B., and Løve Larsen, J.E. (1986) DNA–protein recognition: demonstration of three genetically separated operator elements that are required for repression of the *Escherichia coli deoCABD* promoters by the DeoR repressor. *EMBO J* **5:** 2015–2021.

Voskuil, M.I., Voepel, K., and Chambliss, G.H. (1995) The −16 region, a vital sequence for the utilization of a promoter in *Bacillus subtilis* and *Escherichia coli*. *Mol Microbiol* **17:** 271–279.

van der Vossen, J.M.B., van der Lelie, D., and Venema, G. (1987) Isolation and characterization of *Streptococcus cremoris* WG2-specific promoters. *Appl Environ Microbiol* **53:** 2452–2457.

Wall, L., de Boer, E., and Grosveld, F. (1988) The human β-globin gene 3′ enhancer contains multiple binding sites for an erythroid-specific protein. *Genes Dev* **2:** 1089–1100.

Wells, J.M., Wilson, P.W., Norton, P.W., Gasson, M.J., and LePage, R.W.F. (1993) *Lactococcus lactis*: high-level expression of tetanus toxin fragment C and protection against lethal challenge. *Mol Microbiol* **8:** 1155–1162.

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33:** 103–119.